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DARK FIXATION OF CO_2 BY SUCCULENT LEAVES: CONSERVATION OF THE DARK FIXED CO_2 UNDER DIURNAL CONDITIONS[1,2]

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The characteristic ability of the succulents to incorporate a large net amount of CO_2 in the form of organic acids has been recognized for a long time (9). Although the biochemical mechanisms of this phenomenon have received a great deal of attention, the physiological role has not been fully understood. In the course of our studies on the pathways of the dark fixation of CO_2 (6, 7), it was observed that the $C^{14}O_2$ incorporated in the dark was not lost in the subsequent light period but appeared to be utilized directly in the photosynthesis. From these and other considerations we are impressed with a possibility that the special features of Crassulacean acid metabolism have an adaptive advantage.

MATERIALS AND METHODS

A random sample of young *Bryophyllum calycinum* Salisb. leaves (approximately 2 to 3 cm long, wet weight 0.5 to 0.6 g), picked from the apex of the plant was placed in the apparatus described in a previous communication (6) and permitted to fix $C^{14}O_2$ (generated from 4.6 mg of $BaC^{14}O_3$, specific activity 120 c/mg) for 30 minutes in the dark. Two of the leaves were homogenized immediately with boiling 80% ethanol and an aliquot counted for determination of total activity. All samples were counted at infinite thinness using a Micromil gas flow counter. The total homogenate was then filtered through Whatman no. 1 paper, washed three times with small portions of boiling 80% ethanol, and an aliquot of the total filtrate analyzed by paper chromatography as indicated below. The remaining leaves were removed, placed on moist filter paper, and illuminated with a photoflood lamp (Photospot R.S.P. 2, G.E. 115 to 120 v) placed 31 to 32 inches away. Cool air circulated over the surface of the leaves with a fan to prevent over-heating. At no time did the temperature at the surface of the leaves exceed 28° C. At various intervals pairs of leaves were removed and homogenized in boiling 80% ethanol. The determination of the total activity in the homogenate and the chromatographic analysis of the filtrate was carried out as described below. As a control, one pair of leaves was exposed 30 minutes to $C^{14}O_2$ in the dark, flushed free of $C^{14}O_2$, kept in the dark for 120 minutes, and homogenized in boiling 80% ethanol.

Identification of the labeled photosynthetic and dark products was made on aliquots of the 80% ethanol soluble fractions. This was accomplished by two dimensional chromatography on Whatman no. 1 filter paper using phenol (80) : water (20) (w/w) in the 1st direction and *n*-butanol (74) : acetic acid (19) : water (50) (v/v/v) in the 2nd. Radioautographs were made with "no-screen" x-ray film. In all experiments, non-labeled glucose and sucrose were co-chromatographed with the extract. Identification of the radioactive compounds was made by superposition. Activity was determined directly on the paper with an end-window Geiger tube.

An alternate technique was employed to ascertain whether the $C^{14}O_2$ fixed in the dark was released to

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the atmosphere during the subsequent light period. Matched pairs of leaves were exposed to $C^{14}O_2$ in the dark for 60 minutes. As a control, one pair was immediately homogenized in boiling 80 % ethanol, and the radioactivity determined on an aliquot. The other pair of leaves was placed in a glass chamber and illuminated with three 120-watt incandescent lamps placed 24 inches from the surface of the leaves. During the illumination, moist air was flushed through the chamber and the CO_2 was trapped in 1 *N* NaOH, precipitated as $BaCO_3$, and counted with a gas flow Geiger tube.

Experiments were designed to test the effect of previous conditions of light or dark on the subsequent photosynthetic CO_2 incorporation. Intact plants were placed either in a completely dark room for 17 to 24 hours or in direct sunlight. At the beginning of the experiment matched pairs of leaves were removed, placed in the glass illumination chamber, exposed to $C^{14}O_2$, and allowed to photosynthesize for 30 minutes. The leaves were then homogenized in boiling 80 % ethanol and the total activity measured on an aliquot as described above.

Results and Discussion

The total amount of radioactivity retained by the leaves at each time interval of illumination after the dark incorporation of $C^{14}O_2$ is presented in table I. These data represent the averages of three experiments. A most interesting finding is that the total amount of radiocarbon incorporated in the dark period remained constant during the subsequent light period as well as during the dark control.

In our previous research concerning the dark metabolism of $C^{14}O_2$ in Bryophyllum (7) we demonstrated that no detectable activity was incorporated into either the carbohydrates or their phosphorylated derivatives, all activity being associated with the organic and amino acids. Chromatographic analysis of the extract from the dark control confirmed this. However, when the leaves were transferred to light in the absence of exogenous $C^{14}O_2$ significant activity was associated with the sucrose from the 80 % ethanol soluble fraction. Figure 1 presents the radioautographs from the dark and light extracts. It is clear that upon exposure of the leaf to light, not only was there a conservation of the $C^{14}O_2$ fixed in the dark, but that CO_2 was utilized in the formation of photosynthetic products.

Table I

Radioactivity Retained by Bryophyllum Leaves after Illumination

Duration of light	0	10	30	60	120	(120) Dark control
Total activity in homogenate * (cpm/mg leaf)	737	781	706	750	813	745

* Samples were counted within a statistical error of 5 %.

Table II

Loss of Dark Fixed $C^{14}O_2$ During the Subsequent Light Period

Experiment	Total activity fixed in dark (cpm)	Total free $C^{14}O_2$ trapped as $BaCO_3$ (cpm)	Percent total activity lost
I	2.4×10^5	5.4×10^3	2.2
II	5.0×10^5	9.1×10^3	1.8

Evidence for this conservation of dark-fixed CO_2 during photosynthesis was obtained by the 2nd technique in which the CO_2 was trapped during the process of photosynthesis. The data from typical experiments are shown in table II. It is clear that less than 3 % of the total activity accumulated by the leaves was in equilibrium with the external atmosphere. This experiment indicates that the CO_2 incorporated during the dark was efficiently retained, and utilized during subsequent photosynthesis in the leaf. Similar results have been observed with a marine flagellate, *Dunaliella euchlora* by Ryther (5). Ryther interprets his data as evidence for a preferential utilization of endogenous CO_2.

The ability of succulents to incorporate large amounts of CO_2 in the dark, and subsequently utilize this CO_2 for photosynthesis is a possible biochemical adaptation to arid conditions. The thick spongy leaves and stems with rather impermeable cuticles are useful anatomical structures for water conservation. Since photosynthetic activity takes place at those times when water loss is maximal, re-utilization by the leaf of the dark incorporated CO_2 would tend to minimize the need for opening the stomata to permit gas exchange, and might aid in the conservation of water. Physiological observations of Loftfield (2) support this hypothesis. He demonstrated that, unlike non-succulents, the stomata of the succulents remained closed during the day, but opened at night to permit gas exchange.

Thomas et al (10) have shown that under normal atmospheric CO_2 concentration (0.03 %), light deacidification takes place in succulent leaves. However, when the leaves are exposed to high concentrations of CO_2 (5 to 10 %), this light deacidification can be retarded, and under certain conditions light acidification can be induced. The only known mechanism for the synthesis of carbohydrates from dicarboxylic acids involves decarboxylation and reversal of glycolysis. The observations of Thomas et al (10) could be interpreted as evidence for the preferential carboxylation of ribulose diphosphate as compared to phosphoenol-pyruvate. During the light period following dark acidification CO_2 liberated via decarboxylation immediately enters into the light CO_2 fixation mechanism. However, when the ribulose diphosphate pathway for CO_2 fixation is saturated at high CO_2 concentrations in the light, the acidification system involving phosphoenol-pyruvate carboxylase can be utilized.

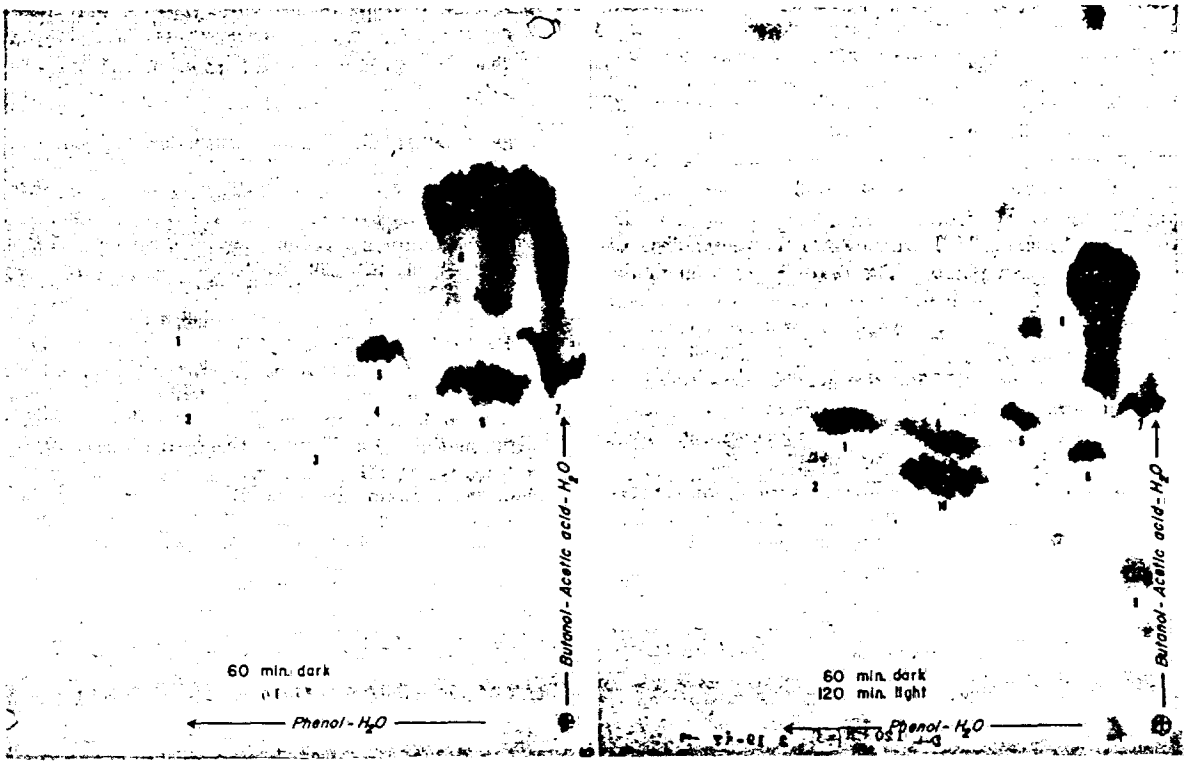

FIG. 1. Radioautograms of two dimensional paper chromatograms of extracts from 60-minute dark fixation of $C^{14}O_2$, and 60-minute dark fixation of $C^{14}O_2$ followed by 120-minute photosynthesis in absence of $C^{14}O_2$. Compounds indicated are: 1. alanine, 2. glutamine, 3. asparagine, 4. glycine-serine, 5. glutamate, 6. aspartate, 7. citrate-isocitrate, 8. malate, 9. phosphoglycerate, and 10. sucrose.

The effects of previous exposure of the leaves to light or dark on the total photosynthetic CO_2 fixation are presented in table III. The experiments were designed so that the leaves of the plants pretreated in the light would be depleted of most of their organic acids. These data are very similar to those presented by Sen and Leopold (8). There are several interpretations that can be made from these results. Sen and Leopold favor the view that there is some temporary impairment of the mechanisms of photosynthesis during the previous exposure to dark. This impairment was manifested in the diminution of the $C^{14}O_2$ incorporated. Another interpretation is possible if one postulates that the rates of photosynthesis subsequent to either pretreatment are approximately the same. The CO_2 fixed during the dark periods would provide a considerable store of endogenous CO_2 which was preferentially utlized. This would result in the decreased utilization of the exogenous CO_2. It is well known from the work of Pucher et al (3, 4) that there is an inverse quantitative relationship between total titratable acids and net carbohydrate stores in succulent leaves subjected to diurnal conditions.

TABLE III

PHOTOSYNTHETIC $C^{14}O_2$ FIXATION LEAVES PRETREATED IN DARK OR IN LIGHT

PRETREATED IN LIGHT		PRETREATED IN DARK		
HOURS OF PRE-TREATMENT	TOTAL CPM	HOURS OF PRE-TREATMENT	TOTAL CPM	RATIO LIGHT/DARK
8	38.2×10^5	17	7.7×10^5	5
8	23.9×10^6	24	1.9×10^6	12

The known pathways for the interconversion of Krebs cycle organic acids and carbohydrates depend upon decarboxylation and the reversal of glycolysis. The labeling experiments of Varner and Burrell (11) and Gibbs (1) completely support this hypothesis. This evidence in conjunction with the experiments presented in this paper indicates that the CO_2 derived from decarboxylation of the organic acids is retained by the cell and metabolized via the normal photosynthetic mechanisms.

SUMMARY

Detached leaves of *Bryophyllum calycinum* are able to fix, in the dark, large amounts of $C^{14}O_2$ in the form of organic and amino acids. When these leaves are exposed to light in the absence of $C^{14}O_2$, radioactivity is found in the carbohydrate fraction. The dark fixed $C^{14}O_2$ is efficiently conserved and metabolized during subsequent photosynthetic reactions.

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